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P. SWAIN

Certifying Officer

GRF ANALOGS WITH INCREASED BIOLOGICAL POTENCY

RELATED APPLICATIONS

This application is a continuation-in-part of applications serial numbers 08/702,113 and 08/702,114 filed on August 23, 1996 which are still pending and which are continuations in part of application 08/651,645 filed on May 22, 1996, which is abandoned and is a continuatión-in-part of application serial number 08/453,067 filed on May 26, 1995 and which is abandoned and all above applications are all incorporated by reference.

BACKGROUND OF THE INVENTION

(a) Field of the Invention

The invention relates to hydrophobic GRF analogs with increased biological potency and prolonged activity, their application as anabolic agents and treatment of growth hormone deficiencies.

(b) Description of Prior Art

Growth hormone (GH) or somatotropin, secreted by the pituitary gland constitute a family of hormones which biological activity is fundamental for the linear growth of a young organism but also for the maintenance of the integrity at its adult state. GH acts directly or indirectly on the peripheral organs by stimulating the synthesis of growth factors (insulin-like growth factor-I or IGF-I) or of their receptors (epidermal growth factor or EGF). The direct action of GH is of the type referred to as anti-insulinic, which favors the lipolysis at the level of adipose tissues. Through its action on IGF-I (somatomedin C) synthesis and secretion, GH stimulate the growth of the cartilage and

the bones (structural growth), the protein synthesis and the cellular proliferation in multiple peripheral organs, including muscles and the skin. Through its biological activity, GH participates within adults in the maintenance of a protein anabolism state, and plays a primary role in the tissue regeneration phenomenon after a trauma.

The decrease of GH secretion with age, demonstrated in humans and animals, favors a metabolic shift towards catabolism which initiates or participates in the aging of an organism. The loss in muscle mass, the accumulation of adipose tissue, the bone demineralization, the loss of tissue regeneration capacity after an injury, which are observed in elderly, correlate with the decrease in the secretion of GH.

GH is thus a physiological anabolic agent absolutely necessary for the linear growth of children and which controls the protein metabolism in adults.

The secretion of GH by the pituitary gland is principally controlled by two hypothalamic peptides, somatostatin and growth hormone-releasing factor (GRF). Somatostatin inhibits its secretion, whereas GRF stimulates it.

The human GH has been produced by genetic engineering for about ten years. Until recently most of the uses of GH were concerned with growth delay in children and now the uses of GH in adults are being studied. The pharmacological uses of GH and GRF may be classified in the following three major categories.

Children growth

Treatments with recombinant human growth hormone have been shown to stimulate growth in children with pituitary dwarfism, renal insufficiencies, Turner's syndrome and short stature. Recombinant human GH is presently commercialized as an "orphan drug" in Europe and in the United States for children's growth retardation caused by a GH deficiency and for children's renal insufficiencies. The other uses are under clinical trial investigation.

Long term treatment for adults and elderly patients

A decrease in GH secretion causes changes in body composition during aging. Preliminary studies of one-year treatment with recombinant human GH reported an increase in the muscle mass and in the thickness of skin, a decrease in fat mass with a slight increase in bone density in a population of aged patients. With respect to osteoporosis, recent studies suggest that recombinant human GH does not increase bone mineralization but it is suggested that it may prevent bone demineralization in post-menopausal women. Further studies are currently underway to demonstrate this theory.

Short term treatment in adults and elderly patients

In preclinical and clinical studies, growth hormone has been shown to stimulate protein anabolism in wound and bone healing in cases of burn, AIDS and cancer.

GH and GRF are also intended for veterinary pharmacological uses. Both GH and GRF stimulate growth in pigs during its fattening period by favoring the

deposition of muscle tissue instead of adipose tissue and increase milk production in cows, and this without any undesired side effects which would endanger the health of the animals, and without any residue in the meat or milk being produced. The bovine somatotropin (BST) is presently commercialized in the United States.

Most of the clinical studies undertaken were conducted with recombinant GH. GRF is considered as a second generation product destined to replace, in the near future, the use of GH in most instances. Accordingly, the use of GRF presents a number of advantages over the use of GH *per se*.

Physiological advantages

Growth hormone (GH) is secreted by the pituitary gland in a pulse fashion. Since this rhythm of secretion is crucial for an optimal biological activity, the administration of GH to correspond to its natural mode of secretion is difficult to achieve. When GRF is administered in a continuous fashion as a slow releasing preparation or as an infusion, it increases GH secretion while respecting its pulsatility.

The recombinant GH which is presently commercialized is the 22 kDa form whereas GRF induces the synthesis and secretion from the pituitary gland of all the chemical isomers of GH which participate in a wider range of biological activities.

A treatment with GH results in a decreased capacity of the pituitary gland to secrete endogenous growth hormone, and the GH response to GRF is diminished after such a treatment. On the contrary, a

treatment with GRF does not present this disadvantage, its trophic action on the pituitary gland increases this gland's secreting capacity in normal animals and in patients with somatotroph insufficiency.

Economical advantages

The production of GH by genetic engineering is very expensive for clinical use. In particular, there are risks of contamination of these commercial preparation with material from the bacterial strain used. These bacterial contaminants may be pyrogens or may result in immunogenic reactions in patients. The purification of the recombinant product is carried out by following a plurality of successive chromatography steps. The drastic purity criteria imposed by regulatory agencies necessitate multiple quality control steps.

On the other hand, the synthesis of GRF is of chemical nature. The synthesis is carried out in a solid phase and its purification is done in a single step using high performance liquid chromatography (HPLC). Also the quantity of GRF to be administered is much less than the quantity of GH for the same biological result.

Even with all these advantages, GRF is still not commercialized as a therapeutic agent to date, mainly because of its instability. The human GRF is a peptide of 44 amino acids of the following sequence:

Tyr	Ala	Asp	Ala	Ile	Phe	Thr	Asn	Ser	Tyr	Arg	Lys	Val	Leu	Gly	Gln
1				5					10					15	
Leu	Ser	Ala	Arg	Lys	Leu	Leu	Gln	Asp	Ile	Met	Ser	Arg	Gln	Gln	Gly
			20					25					30		

Glu Ser Asn Gln Glu Arg Gly Ala Arg Ala Arg Leu-NH_2
35 40 (SEQ ID NO:1).

The minimum active core is hGRF (1-29)NH_2

Tyr Ala Asp Ala Ile Phe Thr Asn Ser Tyr Arg Lys Val Leu Gly Gln
1 5 10 15

Leu Ser Ala Arg Lys Leu Leu Gln Asp Ile Met Ser Arg
20 25 (SEQ ID NO:2).

As for many peptides, hGRF (1-29)NH_2 is rapidly degraded in a serum medium and its metabolites have no residual biological activity. It has been well established that the action of enzymes, namely that of dipeptidylaminopeptidase type IV, in a blood medium results in the hydrolysis of the peptide bond Ala^2-Asp^3 of GRF. This hydrolysis results in a multitude of negative consequences which were the subject of many studies reported in the literature. Essentially, this hydrolysis leads to the formation of truncated peptides of specific activity reduced to less than 1/1000 of the biological activity.

Clinical studies with children and adults have confirmed that natural hGRF (1-44)NH_2 or the active fragment hGRF (1-29)NH_2 are not potent enough to produce equal effects corresponding to those of recombinant GH.

It is well known that the anchoring of hydrophobic groups, such as -NEt_2 at the C-terminal of a peptidic sequence can result in a significantly increased specific activity. In terms of hydrophobicity, these results are contradicted by a fair number recent works such as those of Muranichi (S. Muranichi et al., 1991, *Pharm. Res.*, **8**:649-652) which stress the inefficacy of the lauroyl group as a hydrophobic group

at the N-terminal to create small peptide analogs having the desired biological activity. Hence, the contradictory investigations of the prior art failed to address the issue of finding a more potent GRF analog using hydrophobic residues.

Gaudreau et al. (P. Gaudreau et al., 1992, *J. Med. Chem.*, **35**(10),:1864-1869) describe the affinity of acetyl-, 6-aminohexanoyl-, and 8-aminooctanoyl-GRF(1-29)NH_2 with the rat pituitary receptor. In this report, none of the fatty acid-GRF compounds tested exhibited a higher affinity than hGRF(1-29)NH_2 itself, and the authors concluded that "...modifications to increase the hydrophobic character at the N-terminus of hGRF(1-29)NH_2 do not constitute a suitable approach to increase receptor affinity.".

Coy et al. (D.H. Cow et al., 1987, *J. Med. Chem.*, **30**:219-222) describe an acetyl-GRF peptide with an increased biological activity on a rat model, more particularly on a rat anesthetized with sodium pento-barbital. The *in vitro* GH response by cultured rat pituitary cells was also analyzed. However, these authors did not synthesize and test fatty acid-GRF ana-logs with a carbon chain longer than two (2) carbon atoms (acetyl group) added at the N-terminus region of the GRF and acetyl cannot be considered a hydrophobic group.

Up to now, most of the GRF analogs described (including those of Gaudreau et al. and those of Coy et al.) have been tested in rat models, either *in vitro* or *in vivo*. Since human and rat GRF(1-29)NH_2 are markedly different, the structure-activity relationships of GRF

are different in both species. Therefore, it is not possible to extrapolate results obtained in rats to humans.

Accordingly, it is necessary to design GRF analogs with improved anabolic potency and having a prolonged activity. This increased potency could result from a resistance to serum degradation and/or from hyperagonistic properties.

It would be highly desirable to be provided with GRF analogs with increased anabolic potency.

SUMMARY OF THE INVENTION

One aim of the present invention is to provide new biodegradable GRF analogs with improved biological potency and prolonged activity.

Another aim of the present invention is to provide GRF analogs with increased anabolic potency and prolonged activity, i.e. capable to substantially elevate insulin-like growth factor I (IGF-I) levels when chronically administered in humans and animals.

Another aim of the present invention is to provide a means to render any GRF analog more biologically potent and with a prolonged activity.

Another aim of the present invention is to provide a method of producing active GRF analogs with improved anabolic potency and prolonged activity.

The present invention relates to the preparation of hydrophobic GRF analogs. These chimeric analogs include an hydrophobic moiety (tail), and can be prepared, either by anchoring one or several hydrophobic tails to the GRF, or by substituting one or several amino-acids by a pseudomicellar residue in the

chemical synthesis of GRF. The GRF analogs in accordance with the present invention are characterized in that:

a) These analogs possess an enhanced biological activity; specifically, they are able to markedly increase GH and IGF-I blood levels when administered in an animal model closely related to human. This characteristic is particularly advantageous in that it results in a reduced dosage of an hyperactive compound being administered to the patient, thus improving treatment efficacy and reducing treatment costs.

b) Both natural amino acid and hydrophobic substances, such as fatty acids, are used for the chemical synthesis of the GRF analogs.

c) They present a high biological activity at infinitely small dosages.

d) They remain active for a prolonged period of time, with a high biological activity.

The use of fatty bodies in accordance with the present invention results in GRF analogs which overcome all the drawbacks of the prior art. The GRF analogs of the present invention exhibit an improved anabolic potency with a reduced dosage and have a prolonged activity. Furthermore, the present invention deals with GRF and any of its analogs, truncated or substituted.

In accordance with the present invention there is provided a hydrophobic GRF analog of formula **A**:

$$X \text{———GRF-peptide} \qquad \mathbf{A}$$

wherein;

the GRF peptide is a peptide of formula **B**

A1-**A2**-Asp-Ala-Ile-Phe-Thr-**A8**-Ser-Tyr-Arg-Lys-**A13**-Leu-**A15**-Gln-Leu-**A18**-Ala-Arg-Lys-Leu-Leu-**A24**-**A25**-Ile-**A27**-**A28**-Arg-**A30**-**R_0** (B)

wherein,

A1 is Tyr or His;

A2 is Val or Ala;

A8 is Asn or Ser;

A13 is Val or Ile;

A15 is Ala or Gly;

A18 is Ser or Tyr;

A24 is Gln or His;

A25 is Asp or Glu;

A27 is Met, Ile or Nle;

A28 is Ser or Asn;

A30 is a bond or any amino acid sequence of 1 up to 15 residues;

R_0 is NH_2 or $NH-(CH_2)$**n**$-CONH_2$, with **n**=1 to 12 and;

X is hydrophobic tail anchored via an amide bond and said hydrophobic tail defining a backbone of 5 to 7 atoms;

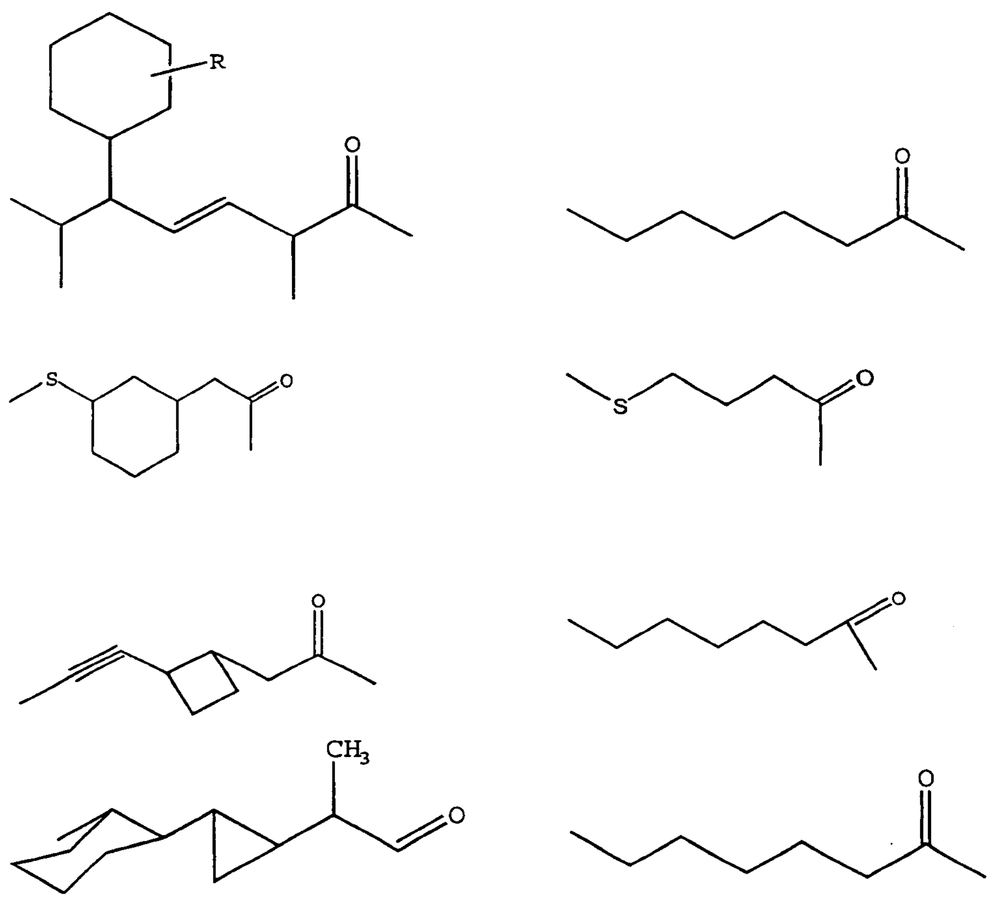
wherein said backbone can be substituted by C_{1-6} alkyl, C_{1-6} cycloalkyl, or C_{6-12} aryl; and comprises at least one rigidifying moiety connected to at least two atoms of the backbone;

said moiety selected from the group consisting of double bond, triple bond, saturated or unsaturated C_{3-9} cycloalkyl, and C_{6-12} aryl.

By the term rigidifying moiety is meant a moiety that will confer rigidity to the hydrophobic tail. The

rigidifying moiety connects at least two atoms which are part of the backbone of the hydrophobic tail. For example, the backbone of the following hydrophic tail is as follows:

Tail	Backbone



Preferably, the backbone is substituted with 2 rigidifying moieties which are independently selected

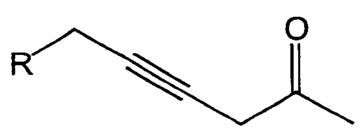
from the group consisting of double bond and saturated or unsaturated C_{3-9} cycloalkyl.

More preferably, the backbone is substituted with 2 rigidifying moieties which are independently selected from the group consisting of double bond, triple bond, saturated C_{3-7} cycloalkyl and C_6 aryl.

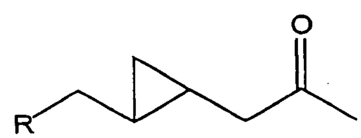
In an alternative embodiment, the backbone is substituted with one rigidifying moiety selected from the group consisting of double bond, triple bond, saturated C_{3-7} cycloalkyl and C_6 aryl.

In an alternative embodiment, the backbone is substituted one rigidifying moiety selected from the group consisting of double bond, triple bond, saturated C_{3-7} cycloalkyl and C_6 aryl, which are located at the 3,4-positions, the 3,5-positions or the 3,6-positions of the backbone.

Preferably, the hydrophobic tail is selected from the group consisting of:

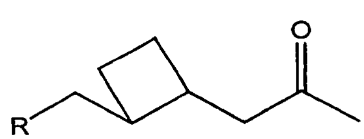


R = H, CH_3, CH_2CH_3



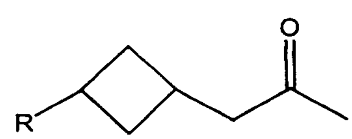
R = H, CH_3, CH_2CH_3

cis or *trans*, both as racemic mixtures or pure enantiomeric pairs.



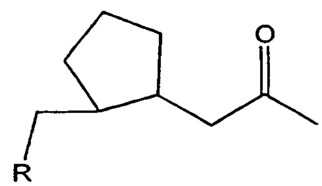
R = H, CH_3, CH_2CH_3

cis or *trans*, both as racemic mixtures or pure enantiomeric pairs.



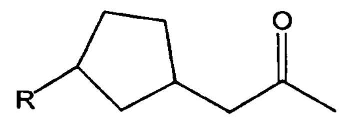
R = H, CH_3, CH_2CH_3

cis or *trans*, (when R$\neq$H)



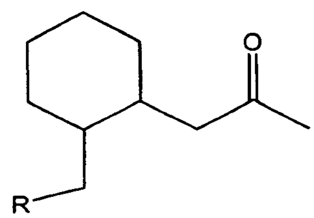
R = H, CH_3, CH_2CH_3

cis or *trans*, both as racemic mixtures or pure enantiomeric pairs.



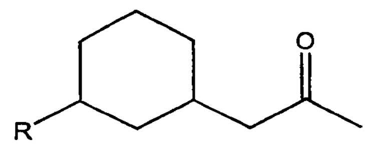
R = H, CH_3, CH_2CH_3

cis or *trans*, (when R≠H) both as racemic mixtures or pure enantiomeric pairs.



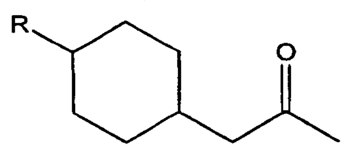
R = H, CH_3, CH_2CH_3

cis or *trans*, both as racemic mixtures or pure enantiomeric pairs.



R = H, CH_3, CH_2CH_3

cis or *trans*, (when R≠H) both as racemic mixtures or pure enantiomeric pairs.



R = H, CH_3

cis or *trans*, (when R≠H)

In accordance with the present invention, there is provided a method of increasing the level of growth hormone in a patient which comprises administering to said patient an effective amount of a GRF analog of the present invention.

In accordance with the present invention, there is provided a method for the diagnosis of growth hormone deficiencies in patients, which comprises adminis-

tering to said patient a GRF analog of the present invention and measuring the growth hormone response.

In accordance with the present invention, there is provided a method for the treatment of pituitary dwarfism or growth retardation in a patient, which comprises administering to said patient an effective amount of a GRF analog of the present invention.

In accordance with the present invention, there is provided a method for the treatment of wound or bone healing in a patient, which comprises administering to said patient an effective amount of a GRF analog of the present invention.

In accordance with the present invention, there is provided a method for the treatment of osteoporosis in a patient, which comprises administering to said patient an effective amount of a GRF analog of the present invention.

In accordance with the present invention, there is provided a method for improving protein anabolism (including protein sparing effect) in human or animal, which comprises administering to said human or animal an effective amount of a GRF analog of the present invention.

In accordance with the present invention, there is provided a method for inducing a lipolytic effect in human or animal afflicted with clinical obesity, which comprises administering to said human or animal an effective amount of a GRF analog of the present invention.

In accordance with the present invention, there is provided a method for the overall upgrading of

somatotroph function in human or animal, which comprises administering to said human or animal an effective amount of a GRF analog of the present invention.

In the present invention the amino acids are identified by the conventional three-letter abbreviations as indicated below, which are as generally accepted in the peptide art as recommended by the IUPAC-IUB commission in biochemical nomenclature:

Alanine	Ala
Arginine	Arg
Asparagine	Asn
Aspartic Acid	Asp
Cysteine	Cys
Glutamic Acid	Glu
Glycine	Gly
Histidine	His
Leucine	Leu
Lysine	Lys
Methionine	Met
Ornithine	Orn
Phenylalanine	Phe
Proline	Pro
Serine	Ser
Threonine	Thr
Tryptophane	Trp
Tyrosine	Tyr
D-Tyrosine	Tyr
Valine	Val

The term "natural amino acid" means an amino acid which occurs in nature or which is incorporated as

an amino acid residue in a naturally occurring peptide. In addition, the abbreviation Nle is intended to mean Norleucine.

Other abbreviations used are:

TFA	Trifluoroacetic acid;
HOBt	1-Hydroxybenzotriazole;
DIC	Diisopropylcarbodiimide;
DMF	Dimethylformamide;
Pip	Piperidine;
DMAP	4-dimethylaminopyridine;
Boc	t-butyloxycarbonyl;
Fmoc	Fluorenylmethyloxycarbonyl;
BOP	Benzotriazo-1-yloxytris (dimethylamino) phos phonium hexafluorophosphate;
Me	Methyl;
HF	Hydrofluoric acid;
NEt_3	Triethylamine; and
TEAP	Triethylammonium phosphate (buffer).

All the peptide sequences set out herein are written according to the generally accepted convention whereby the N-terminal amino acid is on the left and the C-terminal amino acid is on the right.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a graph of the effect of subcutaneously injected hGRF(1-29)NH_2 analogs on pig serum IGF-1;

Fig. 2 is a curve of the effect of one intravenous injection of (4μg/kg) hGRF(1-29)NH_2 and (4μg/kg) (Hexenoyl trans-3)$^{\circ}$ hGRF (1-29)NH_2 (TT-01024) + analog on pig serum GH;

Fig. 3 is a graph showing the effect of various doses of hGRF(1-29)NH_2 vs [hexenoyl trans-3]^o hGRF(1-29)NH_2 (TT-01024) on the GH area under the curve over 300 minutes following I.V. administration (**P<0.01 and ***P<0.001 when compared to the basal period --60 to 0 min-);

Fig. 4 is a curve of the effect of one subcutaneous injection of 5μg/kg hGRF(1-29)NH_2 and (5μg/kg) (Hexenoyl trans-3)^o hGRF (1-29)NH_2 analog on pig serum GH;

Fig. 5 is a graph showing the effect of various doses of hGRF(1-29)NH_2 vs [Hexenoyl trans-3]^o hGRF(1-29)NH_2 (TT-01024) on the GH area under the curve over 420 minutes following S.C. administration (**P<0.01 and ***P<0.001 when compared to the basal period --60 to 0 min-); and

Fig. 6A to 6C illustrate examples of specific synthesis of GRF analogs with preferred radicals R in accordance with the present invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the use of fatty bodies, namely pseudomicellar residues and/or hydrophobic tails, to produce a new family of highly potent, chimeric fatty body-GRF analogs.

In accordance with the present invention, the fatty body-GRF analogs can be chemically synthesized by anchoring one or several hydrophobic tails at the C- and/or the N- terminal portion of GRF or one of its analogs.

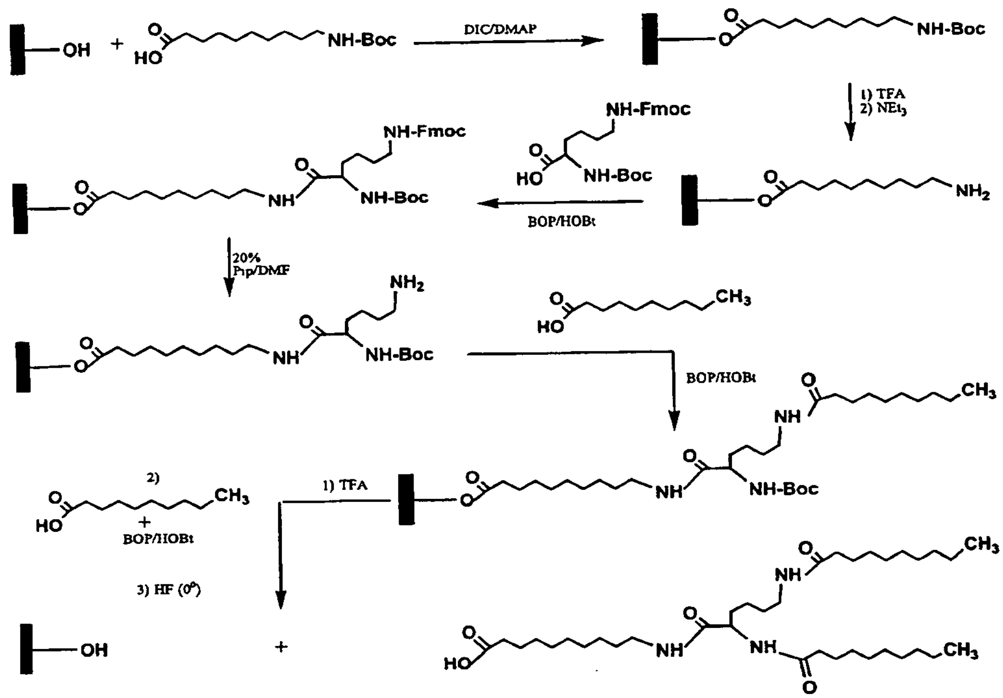
For a better carrying out of the chemical anchoring reaction, hydrophobic functionalized under

the acid form are preferably used. In these conditions, the anchoring reaction is preferably effected in a solid phase (Merrifield R.B., 1963, *J. Am. Chem. Soc.*, **85**:2149; 1964, *J. Am. Chem. Soc.*, **86**:304) using extremely active reagents such as for example Benzotriazole-1-yloxytris (dimethylamino) phosphonium hexafluoro-phosphate known in the prior art (B. Castro et al., 1975, *Tetrahedron letters*, Vol. **14**:1219).

In the case where the hydrophobic tail to be anchored consists in a fatty acid, the activation in view of the anchoring may be carried out *in situ*. Depending on the synthesis strategies used, the peptide anchoring site is liberated just prior to the anchoring in traditional deprotection conditions (Gross et Meienhofer, 1981, *The peptides*, vol. 3, Academic press: pages 1-341). The hydrophobic tail (Ht) is then condensed with the anchoring agent in organic solvents such as an ether (tetrahydrofuranne), an aliphatic halogenated solvent (dichloromethane), a nitrile (acetonitrile) or an amide (dimethylformamide).

With respect to the anchoring dynamic, the preferred working temperatures are between 20 and 60°C. The anchoring reaction time when hydrophobic tail used are more and more hydrophobic, varies inversely with temperature, but varies between 0.1 and 24 hours.

As an illustrative example, the triacyl lysine synthesis as set forth below illustrates in a schematic manner the whole of the anchoring principle of a hydrophobic fatty acid tail.



General GRF analogs synthesis steps were carried out by solid-phase methodology on a 9050™ plus peptide synthesizer (Millipore Corporation, Milford, MA) using Fmoc strategy and synthesis cycles supplied by Millipore. Fmoc amino acids were supplied by Bachem California and other commercials sources. Sequential Fmoc chemistry using BOP/HOBt as coupling methodology were applied to the starting Fmoc-Pal-PEG resin (Millipore, catalog number: GEN 913383) for the production of C-terminal carboxamides. Fmoc deprotections were accomplished with piperidine 20% solution in DMF. After synthesis completion, the resin was well washed with DMF and ether prior to drying. Final cleavages of side chain protecting groups and peptide-resin bonds were performed using Millipore supplied procedure consisting of the following mixture: TFA, water,

phenol, triisopropylsilane (88:5:5:2). Peptides were then precipitated and washed with ether prior to drying. Reverse phase HPLC purification (buffer A: TEAP 2.5; buffer B: 80% CH_3CN in A) using a water pep 4000, absorbance 214nm, detector model 486, flow rate 50ml/min.; linear gradient generally from 25 to 60%B in 105 min.) followed by a desalting step (buffer C:0.1% TFA in H_2O; buffer D:0.1% TFA in CH_3CH/H_2O 80:20) afforded peptides in yields amounting from 10 to 30% with homogeneity greater than 97% as estimated by HPLC (millennium/photodiode array detection).

In accordance with the present invention, the pig was selected as a test specie, since it is a valuable preclinical model for the development of GRF analogs. Indeed, human and porcine GRF(1-29)NH_2 share a 100% homology of structure, and the physiological pattern of GH secretion is almost identical in both species.

Moreover, the potency of the GRF analogs was assessed as their ability to significantly increase IGF-I blood levels rather than their acute GH releasing potency. Indeed, it is known that the anabolic and healing effects of GH or GRF induced GH are mediated by an increase in IGF-I synthesis and secretion. Therefore, the measurement of GRF induced IGF-I elevation is the best indicator of the treatment efficacy.

The present invention will be more readily understood by referring to the following examples which are given to illustrate the invention rather than to limit its scope.

EXAMPLE I

Effect Of repeated administrations of [BUTYRYL^0], [OCTANOYL^0]-, [HEXANOYL^0]-[HEXANOYL^{30}], [HEXANOYL^{0,30}], HGRF(1-29)NH_2 and [HEXANOYL^0] HGRF(1-44)NH_2 VS hGRF(1-29)NH_2 on serum IGF-I levels in pigs

The objective of these experiments was to assess the potential of the GRF analogs as anabolic agents. It is known that GH or GRF-induced GH secretion exert their anabolic effect via an increase in insulin-like growth factor I (IGF-I) synthesis and secretion, that result in elevated levels of circulating IGF-I. It has been previously demonstrated that the intensity of the anabolic response to a GRF analog treatment is proportional to the increase in IGF-I levels in pigs (Dubreuil P. et al., 1990, *J. Anim. Sci.*, **68**:1254-1268).

Therefore, in order to investigate the anabolic potency of the fatty acid-GRF analogs, their ability to increase IGF-I levels following repeated S.C. administrations in pig was evaluated.

Experiment 1

26 Landrace x Yorkshire castrated male pigs (40-45kg BW) were randomly distributed into 4 experimental groups:

1- hGRF(1-29)NH_2 (20μg/kg, n=7)
2- [octanoyl^0] hGRF(1-29)NH_2 (20μg/kg, n=6)
3- [hexanoyl^0] hGRF(1-29)NH_2 (20μg/kg, n=6)
4- [butyryl^0] hGRF(1-29)NH_2 (20μg/kg, n=7)

Each animal was injected BID (twice a day) subcutaneously for 4 consecutive days. One blood sample was collected each morning prior to the first injection

of the day, and the day after the last injection, for IGF-I measurement.

Experiment 2

40 Landrace x Yorkshire castrated male pigs (40-45 kg BW) were randomly distributed into 5 experimental groups:

1- saline (n=8)
2- hGRF(1-29)NH_2 (40μg/kg, n=8)
3- [hexanoyl^0] hGRF(1-29)NH_2 (10μg/kg, n=8)
4- [hexanoyl^0] hGRF(1-29)NH_2 (20μg/kg, n=8)
5- [hexanoyl^0] hGRF(1-29)NH_2 (40μg/kg, n=8)

Each animal was injected BID (twice a day) subcutaneously for 5 consecutive days. One blood sample was collected each morning prior to the first injection of the day, and the day after the last injection, for IGF-I measurement.

Experiment 3

48 Landrace x Yorkshire castrated male pigs (40-45 kg BW) were randomly distributed into 6 experimental groups:

1- Saline (n=8)
2- hGRF(1-44)NH_2 (30μg/kg, n=8)
3- [hexanoyl^0]hGRF(1-44)NH_2 (30μg/kg, n=8)
4- [hexanoyl^0]hGRF(1-29)NH_2 (20μg/kg, n=8)
5- [hexanoyl^{30}]hGRF(1-29)NH_2 (20μg/kg, n=8)
6- [hexanoyl$^{0,\ 30}$]hGRF(1-29)NH_2 (20μg/kg, n=8)

The selected doses were 30μg/kg for hGRF(1-44)NH_2 analogs and 20μg/kg for hGRF(1-29)NH_2 analogs, which give identical doses on a molar basis. Each animal was injected BID (twice a day) subcutaneously for 5

consecutive days. One blood sample was collected each morning prior to the first injection of the day, and the day after the last injection, for IGF-I measurements.

IGF-I measurements

IGF-I levels were measured in pig serum by double antibody radioimmunoassay after formic acid-acetone extraction, as previously described (Abribat T. et al., 1993, *J. Endocrinol.*, 39:583-589). The extraction prior to radioimmunoassay is a necessary step to remove endogenous IGF-binding proteins.

Statistical analysis

In both experiments, the IGF-I data were analyzed by a two way repeated measure analysis of variance, with day and treatment (GRF analog) as sources of variation. Multiple comparison procedures were there run (Student-Newman Keuls method). A $P < 0.05$ was considered as statistically significant.

Results

Experiment 1

There were both a significant effect of day ($P=0.0004$) and a significant treatment x day interaction ($P=0.011$), indicating that the increase in IGF-I levels was dependent on the analog tested (Table 1). Blood samples for IGF-I measurements were collected daily prior to the first injection of compounds. Data are shown as mean ± SEM of 6 to 7 values per group.

Table 1

Effect of repeated SC injection (20μg/kg BID x 4 days) of GRF analogs on serum IGF-I levels

Treatment (BID, 20μg/kg SC)	Day 1 (pretreatment) (ng/ml)	Day 2 (ng/ml)	Day 3 (ng/ml)	Day 4 (ng/ml)	Day 5 (ng/ml)
hGRF(1-29)NH_2	252 ± 28	235 ± 19	263 ± 16	258 ± 17	262 ± 24
[octanoyl^0] hGRF(1-29)NH_2	316 ± 22	287 ± 20	301 ± 37	301 ± 37	318 ± 39
[hexanoyl^0] hGRF(1-29)NH_2	248 ± 20	281 ± 28	299 ± 26	319 ± 22^a	342 ± 21^{a,b}
[butyril^0] hGRF(1-29)NH_2	278 ± 20	281 ± 24	302 ± 26	289 ± 26	293 ± 23

Treatment P=0.42
Day P=0.0004
Treatment x Day P=0.011
^{a} $P < 0.05$ when compared to day 1
^{b} $P < 0.05$ when compared to day 2

Multiple comparisons revealed that only [hexanoyl^0] hGRF(1-29)NH_2 elicited an increase in IGF-I levels, which was significant on days 4 (29%, $P < 0.05$) and 5 (38%, $P < 0.05$). Human GRF(1-29)NH_2 had no effect on IGF-I levels at the dose tested.

Experiment 2

There were both a significant effect of day ($P < 0.0001$) and a significant treatment x day interaction ($P < 0.0001$), indicating that the increase in IGF-I levels was dependent on the analog tested (Table 2). Blood samples for IGF-I measurements were collected daily prior to the first injection of the day. Data are shown as mean ± SEM of 8 values per group.

Table 2

Dose-related effect of repeated SC injection (BID x 5 days) of GRF analogs on serum IGF-I levels

Treatment BID, SC	Day 1 (pretreatment) (ng/ml)	Day 2 (ng/ml)	Day 3 (ng/ml)	Day 4 (ng/ml)	Day 5 (ng/ml)	Day 6 (ng/ml)
saline	282 ± 33	266 ± 30	281 ± 34	293 ± 30	287 ± 32	289 ± 33
hGRF(1-29)NH_2 (40μg/kg)	244 ± 24	243 ± 16	267 ± 20	275 ± 27	267 ± 17	256 ± 15
[hexanoyl^0] hGRF (1-29)NH_2 (10μg/kg)	303 ± 31	327 ± 20	337 ± 25	338 ± 25	366 ± 37[a]	350 ± 34[a]
[hexanoyl^0] hGRF (1-29)NH_2 (20μg/kg)	302 ± 38	341 ± 37	368 ± 43[a]	362 ± 40[a]	362 ± 45[a]	368 ± 57[a]
[hexanoyl^0] hGRF (1-29)NH_2 (40μg/kg)	252 ± 35	275 ± 32	319 ± 31[a]	354 ± 41[a,b]	350 ± 34[a,b]	374 ± 33[a,b,c]

Treatment P=0.23; Day P=0.0001
Treatment x Day P=0.0001
[a] $P < 0.05$ when compared to day 1
[b] $P < 0.05$ when compared to day 2
[c] $P < 0.05$ when compared to day 3

Multiple comparisons revealed that all three tested doses of [hexanoyl^0] hGRF(1-29)NH_2 increased IGF-I levels. At 10μg/kg, IGF-I levels were significantly increased at days 5 and 6 (16 to 21%, $P < 0.05$). At 20μg/kg, they were increased at days 3, 4, 5 and 6 (20 to 22%, $P < 0.05$). At 40μg/kg, they were increased at days 3, 4, 5 and 6 (27 to 48%, $P < 0.05$). The serum IGF-I levels remained stable in saline - and hGRF(1-29)NH_2 - treated pigs.

Finally, a regression analysis revealed that the increase in IGF-I concentrations from day 1 to day 6 was dependent on the dose of [hexanoyl^0]

hGRF(1-29)NH_2 (ΔIGF-I = 11.9 + (2.77 * dose); r = 0.68, P < 0.0001).

Experiment 3

There were both a significant effect of day (P<0.0001) and a significant treatment x day interaction (P<0.0001), indicating that the increase in IGF-I levels was dependent on the analog tested (Table IV). Multiple comparison revealed that analogs with an hexanoyl function branched at the N-terminal region of GRF were highly potent:

- [hexanoyl^0] hGRF(1-29)NH_2 significantly increased IGF-I levels on days 5 and 6 (by 28% and 31%, P<0.05)
- [hexanoyl$^{0, 30}$] hGRF(1-29)NH_2 significantly increased IGF-I levels on days 4, 5 and 6 (by 32%, 35% and 43%, P<0.05)
- [hexanoyl^0] hGRF(1-44)NH_2 significantly increased IGF-I levels on days 3, 4, 5 and 6 (by 41%, 54%, 50% and 61%, P<0.05)

As previously observed for hGRF(1-29)NH_2 (experiments 1 and 2), the full length hGRF(1-44)NH_2 had little or no effect on IGF-I levels (except for a significant effect on day 5, which was not sustained on day 6). Finally, the anchoring of an hexanoyl function at the C-terminal region of hGRF(1-29)NH_2 yielded an analog with increased potency when compared to hGRF(1-29)NH_2 (21% increased in IGF-I levels on day 6, P<0.05), but less potent than [hexanoyl^0]hGRF(1-29)NH_2.

Human GRF(1-29)NH_2 and hGRF(1-44)NH_2 were injected at 20μg/kg and 30μg/kg, respectively, in order

to achieve equimolar concentrations. Data shown are mean ± SEM of 8 values per group.

Table 3

Effect of multiple SC injections of GRF analogs (BID x 5 days) on serum IGF-I levels in growing pigs

Treatment BID, SC	Day 1 (pretreatment) (ng/ml)	Day 2 (ng/ml)	Day 3 (ng/ml)	Day 4 (ng/ml)	Day 5 (ng/ml)	Day 6 (ng/ml)
saline	215 ± 21	215 ± 28	219 ± 25	226 ± 28	249 ± 30	234 ± 24
hGRF(1-44)NH_2 (30μg/kg)	245 ± 21	254 ± 22	285 ± 26	297 ± 28	303 ± 26[a]	296 ± 26
[hexanoyl^0] hGRF(1-29)NH_2 (20μg/kg)	272 ± 45	292 ± 52	292 ± 57	315 ± 57	347 ± 44[a,b,c]	356 ± 44[a,b,c]
[hexanoyl^{30}] hGRF(1-29)NH_2 (20μg/kg)	297 ± 30	270 ± 25	287 ± 24	278 ± 18	276 ± 20	327 ± 24[b]
[hexanoyl^{0,30}] hGRF(1-29)NH_2 (20μg/kg)	205 ± 24	212 ± 26	253 ± 33	271 ± 36[a,b]	277 ± 29[a,b]	294 ± 26[a,b]
[hexanoyl^0] hGRF(1-44)NH_2 (30μg/kg)	241 ± 30	290 ± 33	340 ± 41[a]	372 ± 40[a,b]	361 ± 46[a,b]	388 ± 49[a,b,c]

Treatment P=0.16
Day P<0.0001
Treatment x Day P<0.0001

[a] $P < 0.05$ when compared to day 1
[b] $P < 0.05$ when compared to day 2
[c] $P < 0.05$ when compared to day 3

Conclusions

Neither hGRF(1-29)NH_2 nor hGRF(1-44)NH_2 at doses ranging from 20 to 40 μg/kg were able to modulate IGF-I levels. However, the anchoring of fatty acid rendered GRF more potent and yielded analogs with markedly improved activity on IGF-I secretion. The anchoring of fatty acids was efficient in improving the anabolic potency of both hGRF(1-29)NH_2 and hGRF(1-44)NH_2. From the above results, it is concluded that the ideal fatty acid to use is hexanoic acid or any C6 fatty derivative, and that it should be preferably anchored

at the N-terminal region of GRF to yield maximally potent analogs.

EXAMPLE II

Comparative effects of GRF analogs on IGF-I levels in pigs

This was a 5-day treatment, twice a day S.C. administration of one single dose of each test article vs saline. This experiment was conducted to compare the efficacy of (Aminohexanoyl)$_0$ hGRF (1-29) NH_2, (Hexylformiate)$_0$ hGRF (1-29) NH_2, (Hexenoyl trans-2)$_0$ hGRF (1-29) NH_2, (Hexenoyl trans-3)$_0$ hGRF (1-29) NH_2 and (Muconoyl)$_0$ hGRF (1-29) NH_2 to that of (Hexanoyl)$_0$ hGRF (1-29) NH_2.

All tested compounds belong to the same family of GRF analogs: they are a combination of the natural GRF and natural fatty acids, designed to improve the activity of the molecule.

Identity of tested analogs:		in saline
TT-01015	(Hexanoyl)$_0$ hGRF (1-29) NH_2	20 µg/kg
TT-01021	(Aminohexanoyl)$_0$ hGRF (1-29) NH_2	20 µg/kg
TT-01022	(Hexylformiate)$_0$ hGRF (1-29) NH_2	20 µg/kg
TT-01023	(Hexenoyl trans-2)$_0$ hGRF (1-29) NH_2	20 µg/kg
TT-01024	(Hexenoyl trans-3)$_0$ hGRF (1-29) NH_2	20 µg/kg
TT-01025	(Muconoyl)$_0$ hGRF (1-29) NH_2	20 µg/kg

Route and frequency of test article

ADMINISTRATION:	Two daily subcutaneous injections.
TEST SYSTEM:	Landrace x Yorkshire pigs.
ANIMAL DESCRIPTION:	Fifty six (56) growing barrows pigs weighing 35 kg at the time of purchase.

RATION: Commercial feed concentrate (18% protein) offered ad libitum.

EXPERIMENTAL DESIGN: Fifty six (56) pigs were randomly distributed into 7 experimental groups (n = 8 pigs per group). Each group received two daily S.C. administration of the following treatments (volume: 3 ml, S.C. injection).

group 1: saline 2 x/day
group 2: TT-01015 20 µg/kg 2 x/day
group 3: TT-01021 20 µg/kg 2 x/day
group 4: TT-01022 20 µg/kg 2 x/day
group 5: TT-01023 20 µg/kg 2 x/day
group 6: TT-01024 20 µg/kg 2 x/day
group 7: TT-01025 20 µg/kg 2 x/day

Treatments were administered from day 1 to 5. Immediately before the injections, one blood sample were collected from each animal, and additional blood samples were collected on day 6.

Blood samples were allowed to clot, serum was harvested by centrifugation and submitted to IGF-I assays.

Results are shown in Fig. 1 as D-IGF-I, which is defined as the increase in IGF-I levels from day 1 (pretreatment levels) to day 6 (after 5 days of GRFs administrations). Among all analog tested, only hexanoyl-, hexylformiate-, hexenoyl trans2- and hexenoyl trans3-hGRF(1-29)NH_2 significantly increased IGF-I levels over the 6-day study period, whereas aminohexanoyl- and muconoyl-hGRF(1-29)NH_2 did not. Since

$hGRF(1-29)NH_2$ has been shown to be ineffective at the same dose in the same conditions in previous assays (see Example I), these results show that the addition of various C6 carbon chains at the N-terminus region of GRF increases its bioactivity.

EXAMPLE III

Intravenous GH-releasing potency of (Hexenoyl trans-3)$_0$ hGRF (1-29) NH_2 vs $hGRF(1-29)NH_2$ in pigs

This experiment was conducted to test the I.V. acute GH-releasing potency of (Hexenoyl trans-3)$_0$ hGRF (1-29) NH_2, a GRF analog, in a model physiologically close to human and to compare it to that of $hGRF(1-29)NH_2$.

(Hexenoyl trans-3)$_0$ hGRF (1-29) NH_2 is a combination of the natural $hGRF(1-29)NH_2$ and natural fatty acids. This study was a multidose, single I.V. injection study.

Identity of tested analogs:

TT-01024 (Hexenoyl trans-3)$_0$ hGRF (1-29) NH_2	0.25 µg/kg
TT-01024 (Hexenoyl trans-3)$_0$ hGRF (1-29) NH_2	1 µg/kg
TT-01024 (Hexenoyl trans-3)$_0$ hGRF (1-29) NH_2	4 µg/kg
$hGRF(1-29)NH_2$	0.25 µg/kg
$hGRF(1-29)NH_2$	1 µg/kg
$hGRF(1-29)NH_2$	4 µg/kg

Route and frequency of test article

ADMINISTRATION:	intravenous acute injection.
TEST SYSTEM:	Landrace x Yorkshire pigs.

ANIMAL DESCRIPTION: Fifty six (56) growing barrows pigs weighing 35 kg at the time of purchase.

RATION: Commercial feed concentrate (18% protein) offered ad libitum.

EXPERIMENTAL DESIGN: Fifty (56) pigs (4 spare animals) were cannulated (a catheter surgically implanted in one jugular vein) within on week, before the study. On days 1 and 7, cannulated animals were randomly distributed into 7 groups (n = 4 pigs per group).

group 1:	saline	
group 2:	TT-01024	0.25 μg/kg
group 3:	TT-01024	1 μg/kg
group 4:	TT-01024	4 μg/kg
group 5:	hGRF(1-29)NH_2	0.25 μg/kg
group 6:	hGRF(1-29)NH_2	1 μg/kg
group 7:	hGRF(1-29)NH_2	4 μg/kg

Blood samples for pGH assay were collected every 20 min from 1 hour before to 5 hours after GRF injections, with additional samplings 10 and 30 min after injection (n = 21 samples). Blood samples are allowed to clot at +4°C. Serum will be harvested by centrifugation, stored at -20°C and submitted to pGH assays.

Results are illustrated in Figs. 2 and 3. As shown in Fig. 2, hGRF(1-29)NH_2 (4 μg/kg) induced a rapid GH release that was sustained for approximately 60 minutes following injection. In contrast, hexenoyl trans3-hGRF(1-29)NH_2 injected at the same dose

increased GH levels over a longer period, approximately 260 minutes. In addition, the GH response in the first 60 minutes was moderate, suggesting that this analog acts as a GRF, being processed in serum into native GRF in the minutes or hours following injection. As shown in Fig. 3, which presents the effects of various doses of GRF and the analog on the GH area under the curve (0 to 300 minutes following injection), hGRF(1-29)NH_2 produced a significant effect on GH secretion at 4μg/kg, but not at 0.25 or 1 μg/kg, whereas hexenoyl trans3-hGRF(1-29)NH_2 elicited a significant response at all 3 doses tested. In conclusion, these results show that hexenoyl trans3-hGRF(1-29)NH_2 is a GRF analog with increased potency on GH secretion, and suggest that it may act as a GRF, being protected from enzymatic degradation in serum.

EXAMPLE IV

Subcutaneous GH-releasing potency of (Hexenoyl trans-3)$_0$ hGRF (1-29) NH_2 vs hGRF(1-29)NH_2 in pigs

This experiment was conducted to test the S.C. acute GH-releasing potency of (Hexenoyl trans-3)$_0$ hGRF (1-29) NH_2, a GRF analog, in a model physiologically close to human and to compare it to that of hGRF(1-29)NH_2.

Identity of tested analogs:

TT-01024 (Hexenoyl trans-3)$_0$ hGRF (1-29) NH_2 0.31 µg/kg

TT-01024 (Hexenoyl trans-3)$_0$ hGRF (1-29) NH_2 1.25 µg/kg

TT-01024 (Hexenoyl trans-3)$_0$ hGRF (1-29) NH_2 5 µg/kg

TT-01024 (Hexenoyl trans-3)$_0$ hGRF (1-29) NH_2 20 µg/kg

hGRF(1-29)NH_2 1.25 µg/kg

hGRF(1-29)NH_2	5 μg/kg
hGRF(1-29)NH_2	20 μg/kg

Route and frequency of test article

ADMINISTRATION: Subcutaneous acute injection.

TEST SYSTEM: Landrace x Yorkshire pigs.

ANIMAL DESCRIPTION: Sixty four (64) growing barrows pigs weighing 35 kg at the time of purchase.

RATION: Commercial feed concentrate (18% protein) offered ad libitum.

EXPERIMENTAL DESIGN: Thirty six (36) pigs (4 spare animals) were cannulated (a catheter surgically implanted in one jugular vein) within one week, before the study. On days 1 and 7, cannulated animals were randomly distributed into 8 groups (n = 4 pigs per group).

group 1: saline	
group 2: TT-01024	0.31 μg/kg
group 3: TT-01024	1.25 μg/kg
group 4: TT-01024	5 μg/kg
group 5: TT-01024	20 μg/kg
group 6: hGRF(1-29)NH_2	1.25 μg/kg
group 7: hGRF(1-29)NH_2	5 μg/kg
group 8: hGRF(1-29)NH_2	20 μg/kg

Blood samples for pGH assay were collected every 20 min from 1 hour before to 7 hours after GRF injections, (n = 25 samples). Blood samples were allowed to clot at +4CC. Serum is harvested by cen-

trifugation, stored at -20°C and submitted to pGH assays.

Results are shown in Figs. 4 and 5. As shown in Fig. 4, the subcutaneous injection of 5 $\mu g/kg$ hGRF(1-29)NH_2 induced a GH response in the first 60 minutes following administration, whereas the same injection of hexenoyl trans3-hGRF(1-29)NH_2 induced a GH response that was sustained for 240 minutes. The Fig. 5 illustrates the effect of various doses of the GRFs tested on the GH area under the curve over the study period, i.e. from 0 to 420 minutes following injection. Over this period, hGRF(1-29)NH_2 did not induce any significant GH response at any of the tested doses, whereas hexenoyl trans3-hGRF(1-29)NH_2 elicited significant increases of the GH AUC at 5 and 20 $\mu g/kg$. Altogether, these results suggest that hexenoyl trans3-hGRF(1-29)NH_2 is a highly potent GH secretagogue, even when subcutaneously administered.

<u>EXAMPLE V</u>

In accordance with a preferred embodiment of the present invention there is provided a hydrophobic GRF analog of formula **A**:

X ——GRF-peptide **A**

wherein;

the GRF peptide is a peptide of formula **B**

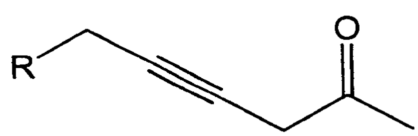
A1-**A2**-Asp-Ala-Ile-Phe-Thr-**A8**-Ser-Tyr-Arg-Lys-**A13**-Leu-**A15**-Gln-Leu-**A18**-Ala-Arg-Lys-Leu-Leu-**A24**-**A25**-Ile-**A27**-**A28**-Arg-**A30**-$\mathbf{R_0}$ **(B)**

wherein,

A1 is Tyr or His;

A2 is Val or Ala;
A8 is Asn or Ser;
A13 is Val or Ile;
A15 is Ala or Gly;
A18 is Ser or Tyr;
A24 is Gln or His;
A25 is Asp or Glu;
A27 is Met, Ile or Nle;
A28 is Ser or Asn;
A30 is a bond or any amino acid sequence of 1 to 15 residues;
R_0 is NH_2 or $NH-(CH_2)$**n**$-CONH_2$, with **n**=1 to 12 and;

X is cis or trans $CH_3-CH_2-CH=CH-CH_2-CO-$, or one element selected from a cis or a trans enantiomer or a racemic mixture of:



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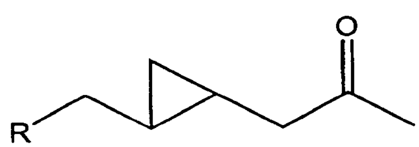

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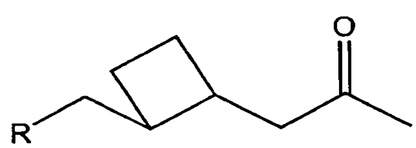

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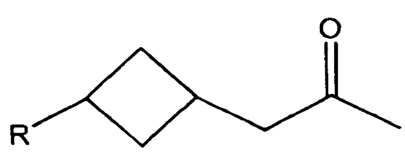

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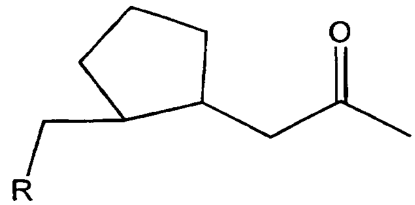

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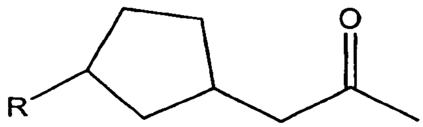

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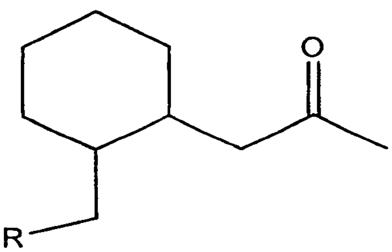

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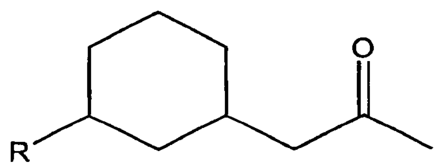

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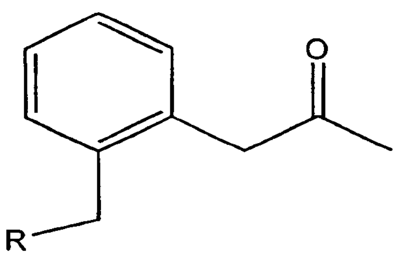

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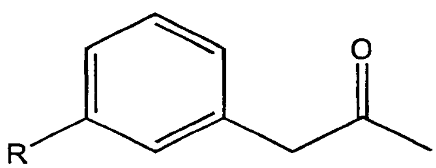

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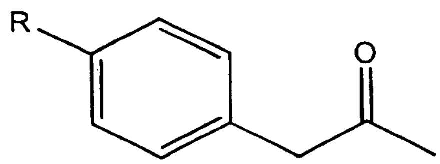

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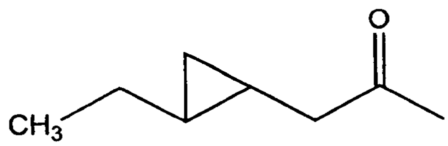
$$CH_3CH_2—C\equiv C—CH_2—CO$$

(13)



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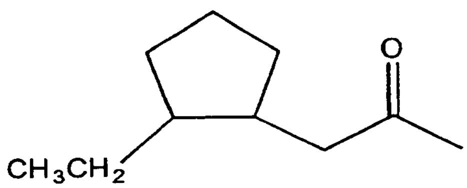

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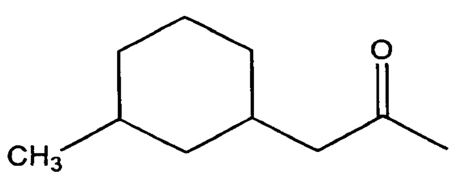

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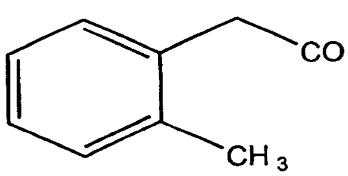

(17)

wherein R is a hydrogen or a lower alkyl.

Fig. 6A to 6C illustrate examples of specific synthesis of GRF analogs with preferred radicals R in accordance with the present invention.

While the invention has been described in connection with specific embodiments thereof, it will be

understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: BRAZEAU, Paul
GRAVEL, Denis

(ii) TITLE OF INVENTION: GRF ANALOGS WITH INCREASED BIOLOGICAL POTENCY

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Evenson, McKeown, Edwards & Lenahan
(B) STREET: Suite 700, 1200 G Street, N.W.
(C) CITY: Washington
(D) STATE: D.C.
(E) COUNTRY: U.S.A.
(F) ZIP: 20005

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/453,067
(B) FILING DATE: 26-MAY-1995

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/651,645
(B) FILING DATE: 22-MAY-1996

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/702,113
(B) FILING DATE: 23-AUG-1996

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/702,114
(B) FILING DATE: 23-AUG-1996

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: EVANS, Joseph D.
(B) REGISTRATION NUMBER: 31,824

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (202) 628-8800
(B) TELEFAX: (202) 628-8844
(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 44 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
Tyr Ala Asp Ala Ile Phe Thr Asn Ser Tyr Arg Lys Val Leu Gly Gln
1               5                   10                  15

Leu Ser Ala Arg Lys Leu Leu Gln Asp Ile Met Ser Arg Gln Gln Gly
            20                  25                  30

Glu Ser Asn Gln Glu Arg Gly Ala Arg Ala Arg Leu
        35                  40
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Tyr Ala Asp Ala Ile Phe Thr Asn Ser Tyr Arg Lys Val Leu Gly Gln
1               5                   10                  15

Leu Ser Ala Arg Lys Leu Leu Gln Asp Ile Met Ser Arg
            20                  25
```

WE CLAIM:

1. An hydrophobic GRF analog of formula **A**:

X ——GRF-peptide **A**

wherein;

the GRF peptide is a peptide of formula **B**

A1-**A2**-Asp-Ala-Ile-Phe-Thr-**A8**-Ser-Tyr-Arg-Lys-**A13**-Leu-**A15**-Gln-Leu-**A18**-Ala-Arg-Lys-Leu-Leu-**A24**-**A25**-Ile-**A27**-**A28**-Arg-**A30**-$\mathbf{R_0}$ (**B**)

wherein,

A1 is Tyr or His;

A2 is Val or Ala;

A8 is Asn or Ser;

A13 is Val or Ile;

A15 is Ala or Gly;

A18 is Ser or Tyr;

A24 is Gln or His;

A25 is Asp or Glu;

A27 is Met, Ile or Nle;

A28 is Ser or Asn;

A30 is a bond or any amino acid sequence of 1 up to 15 residues;

$\mathbf{R_0}$ is NH_2 or $NH\text{-}(CH_2)\mathbf{n}\text{-}CONH_2$, with **n**=1 to 12 and;

X is hydrophobic tail anchored via an amide bond and said hydrophobic tail defining a backbone of 5 to 7 atoms;

wherein said backbone can be substituted by C_{1-6} alkyl, C_{1-6} cycloalkyl, or C_{6-12} aryl; and comprises at least one rigidifying moiety connected to at least two atoms of the backbone;

said moiety selected from the group consisting of double bond, triple bond,

saturated or unsaturated
C_{3-9} cycloalkyl, and C_{6-12} aryl.

2. A pharmaceutical formulation for inducing growth hormone release which comprises as an active ingredient a GRF analog as claimed in claim 1, in association with a pharmaceutically acceptable carrier, excipient or diluent.

3. A method of increasing the level of growth hormone in a patient which comprises administering to said patient an effective amount of a GRF analog as claimed in claim 1.

4. A method for the diagnosis of growth hormone deficiencies in patients, which comprises administering to said patient a GRF analog as claimed in claim 1 and measuring the growth hormone response.

5. A method for the treatment of pituitary dwarfism or growth retardation in a patient, which comprises administering to said patient an effective amount of a GRF analog as claimed in claim 1.

6. A method for the treatment of wound or bone healing in a patient, which comprises administering to said patient an effective amount of a GRF analog as claimed in claim 1.

7. A method for the treatment of osteoporosis in a patient, which comprises administering to said patient

an effective amount of a GRF analog as claimed in claim 1.

8. A method for improving protein anabolism in human or animal, which comprises administering to said human or animal an effective amount of a GRF analog as claimed in claim 1.

9. A method for inducing a lipolytic effect in human or animal inflicted with clinical obesity, which comprises administering to said human or animal an effective amount of a GRF analog as claimed in claim 1.

10. A method for the overall upgrading of somatroph function in human or animal, which comprises administering to said human or animal an effective amount of a GRF analog as claimed in claim 1.

ABSTRACT OF THE INVENTION

The present invention relates to chimeric fatty body-GRF analogs with increased biological potency, their application as anabolic agents and in the diagnosis and treatment of growth hormone deficiencies. The chimeric fatty body-GRF analogs include an hydrophobic moiety (tail), and can be prepared, either by anchoring at least one hydrophobic tail to the GRF, in the chemical synthesis of GRF. The GRF analogs of the present invention are biodegradable, non-immunogenic and exhibit an improved anabolic potency with a reduced dosage and prolonged activity.

Combined Declaration for Patent Application and Power of Attorney

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe that I am the original, first and sole inventor (if only one name listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled GRF ANALOGS WITH INCREASED BIOLOGICAL POTENCY, the specification of which

[X] is attached hereto.

[ ] was filed on ______ as Application No. ______
and (if applicable) was amended on ______

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, § 1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having filing date before that of the application on which priority is claimed;

Prior Foreign Application(s)

Number	Country	Day/Month/Year Filed	Priority Claimed

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States Application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Application Serial No.	Day/Month/Year Filed	Status (Patented, Pending, Abandoned)
08/702,113	23/08/1996	Pending
08/702,114	23/08/1996	Pending
08/651,645	22/05/1996	Abandoned
08/453,067	26/05/1995	Abandoned

I hereby appoint the following attorneys, with full power of substitution, association, and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.
ROBERT MITCHELL, Registration No. 25,007, GUY HOULE, Registration No. 24, 971, PAUL MARCOUX, Registration No. 24,990, KEVIN P. MURPHY, Registration No. 26,674; ROBERT CARRIER, Registration No. 30,726; MICHEL J. SOFIA; Registration No. 37,017; FRANCE CÔTÉ, Registration No. 37,037; JOSEPH D. EVANS, Registration No. 26,269; HERBERT I. CANTOR; Registration No. 24,392; DONALD D. EVENSON, Registration No. 26,160; and GARY R. EDWARDS, Registration No. 31,824 and address all correspondence to:

Evenson, McKeown, Edwards & Lenahan
Suite 700, 1200 G Street, N.W.
Washington, D.C. 20005
U.S.A.

Direct all telephone calls to J.D. Evans at (202) 628-8800

I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application of any patent issued thereon.

Full name of second inventor	Inventor's signature	Date
Paul BRAZEAU		2nd Sept 98
Post Office Address and Residence: 4054 Ave. du Parc Lafontaine, Montréal, Québec, Canada H2L 3M8	Citizenship: Canadian	
Full name of second inventor: Denis GRAVEL	Inventor's signature	Date: Sept. 1st, 1998
Post Office Address and Residence: 207 Des Pyrénées st., St-Lambert, Québec, Canada J4S 1L3	Citizenship: Canadian	

Applicant or Patentee: Paul BRAZEAU et al..
Serial or Patent No.: ______ Atty. Dkt. No.: ______
Filed or Issued: ______
For: GRF ANALOGS WITH INCREASED BIOLOGICAL POTENCY

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS [37 CFR 1.9(f) AND 1.27 (c)] - SMALL BUSINESS CONCERN

I hereby declare that I am

( ) the owner of the small business concern identified below;
(X) an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN THERATECHNOLOGIES INC.
ADDRESS OF CONCERN 630 Blvd. René Lévesque West, 5th floor, Montréal, Québec, Canada H3B 1S6

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 CFR 121.3-18, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention, entitled GRF ANALOGS WITH INCREASED BIOLOGICAL POTENCY by inventor(s) Paul BRAZEAU and Denis GRAVEL described in:

(X) the specification filed herewith;
( ) application serial no. ______, filed ______;
( ) patent no. ______, issued ______.

If the rights held by the above identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor who could not qualify as an independent inventor under 37 CFR 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e). *NOTE· Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. [37 CFR 1.27]

NAME ______
ADDRESS ______
( ) INDIVIDUAL ( ) SMALL BUSINESS CONCERN ( ) NONPROFIT ORGANIZATION

NAME ______
ADDRESS ______
( ) INDIVIDUAL ( ) SMALL BUSINESS CONCERN ( ) NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. [37 CFR 1.28(b)]

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING André de Villers
TITLE OF PERSON OTHER THAN OWNER President and Director - Research & Development
ADDRESS OF PERSON SIGNING 630 Blvd. René Lévesque West, Montréal, Québec, Canada H3B 1S6

SIGNATURE André de Villers DATE 11/09/1998

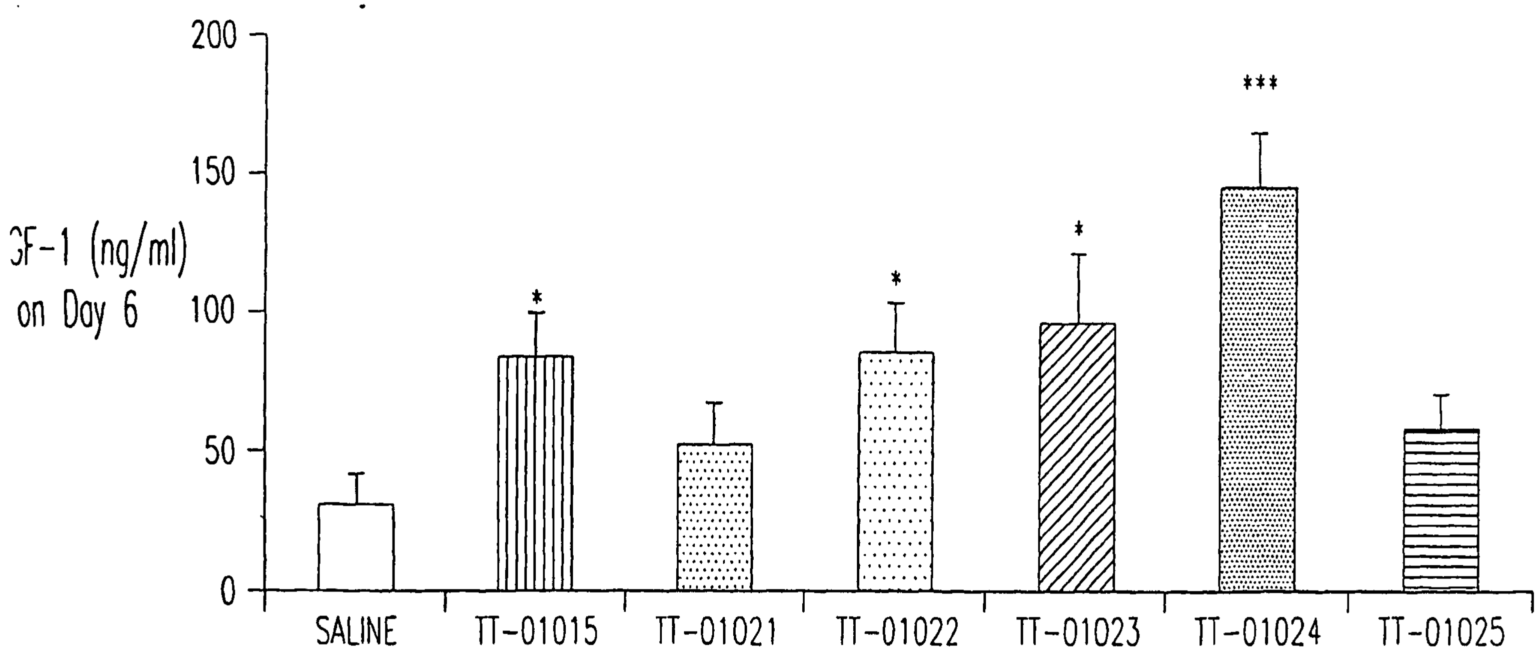

IGF-1 (ng/ml)
on Day 6
200
150
100
50
0
***
*
SALINE
TT-01015
TT-01021
TT-01022
TT-01023
TT-01024
TT-01025

FIG. 1

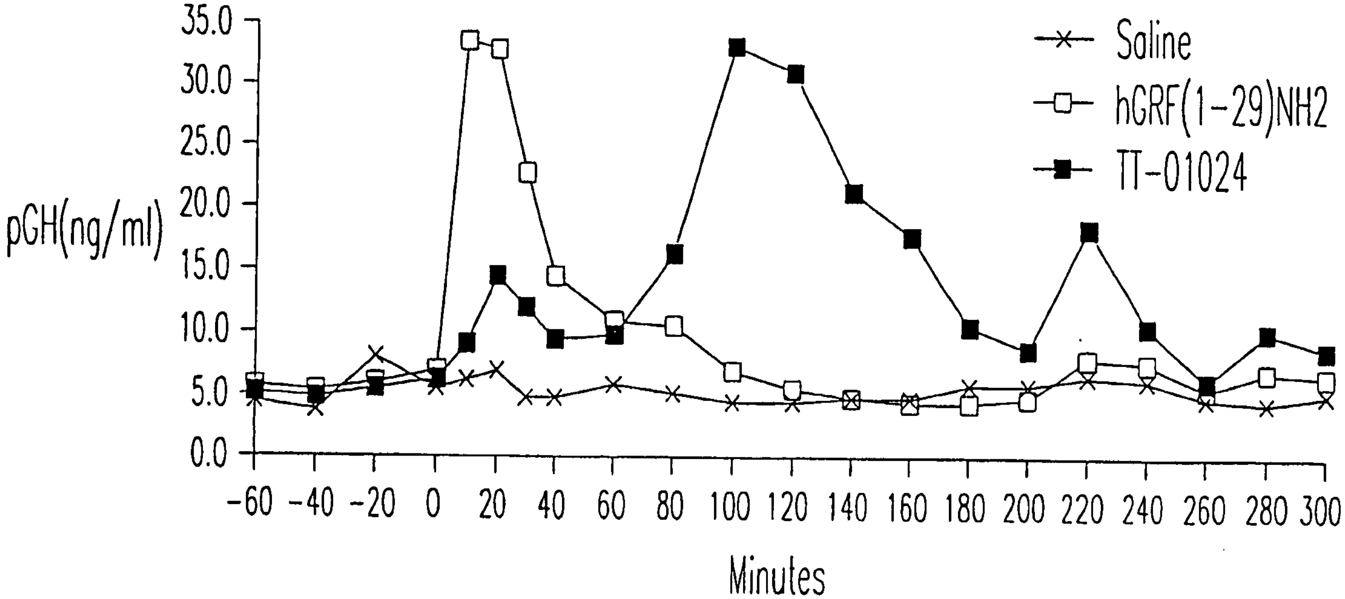

pGH(ng/ml)
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
-60 -40 -20 0 20 40 60 80 100 120 140 160 180 200 220 240 260 280 300
Minutes
Saline
hGRF(1-29)NH2
TT-01024

FIG. 2

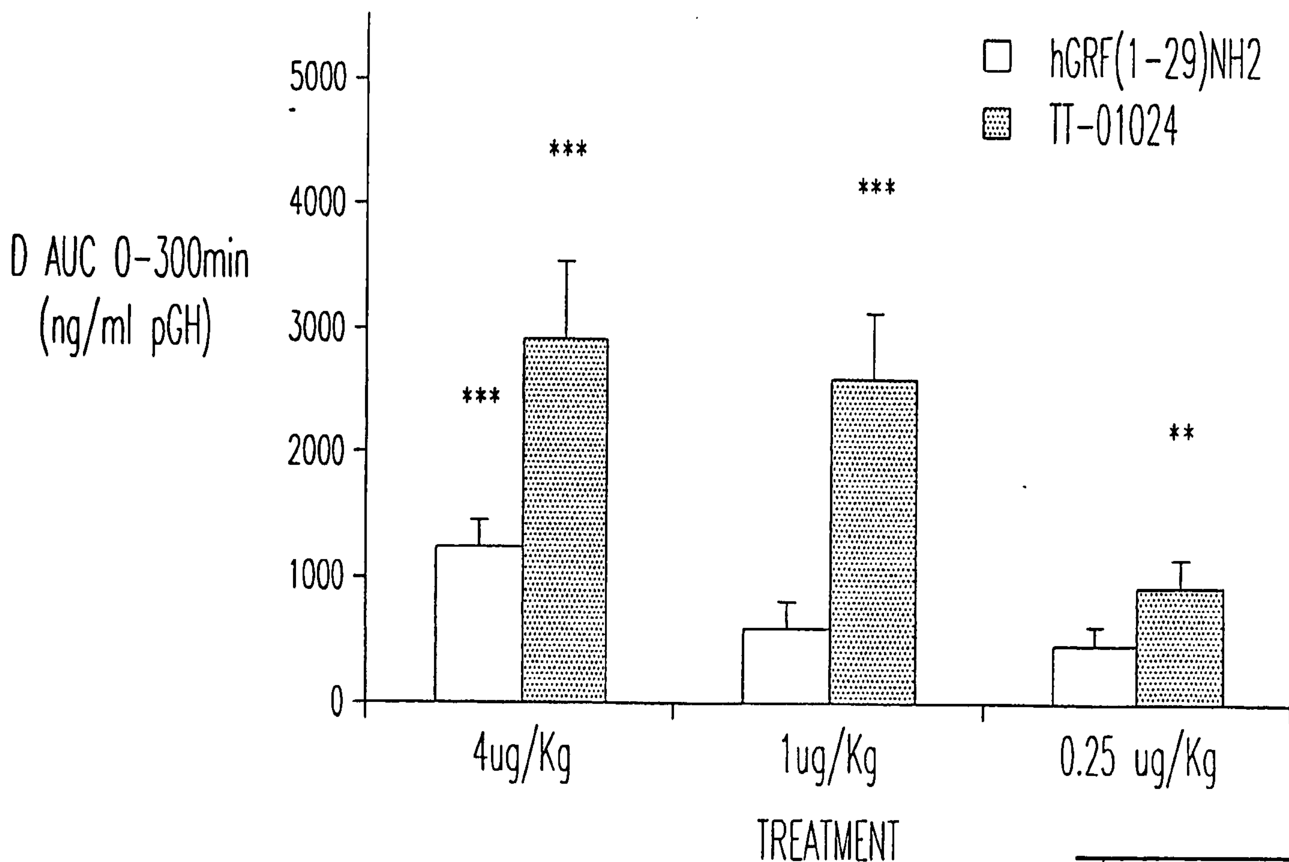
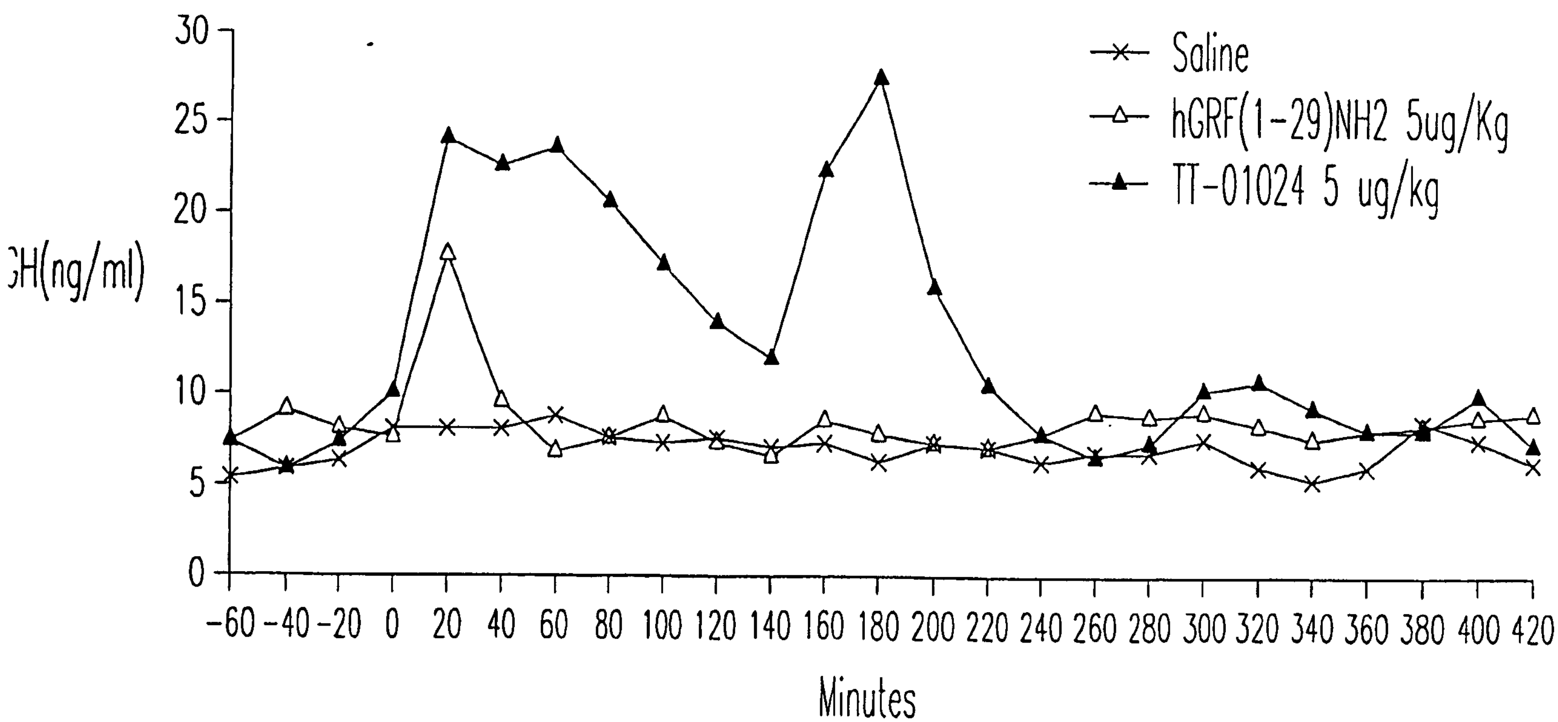
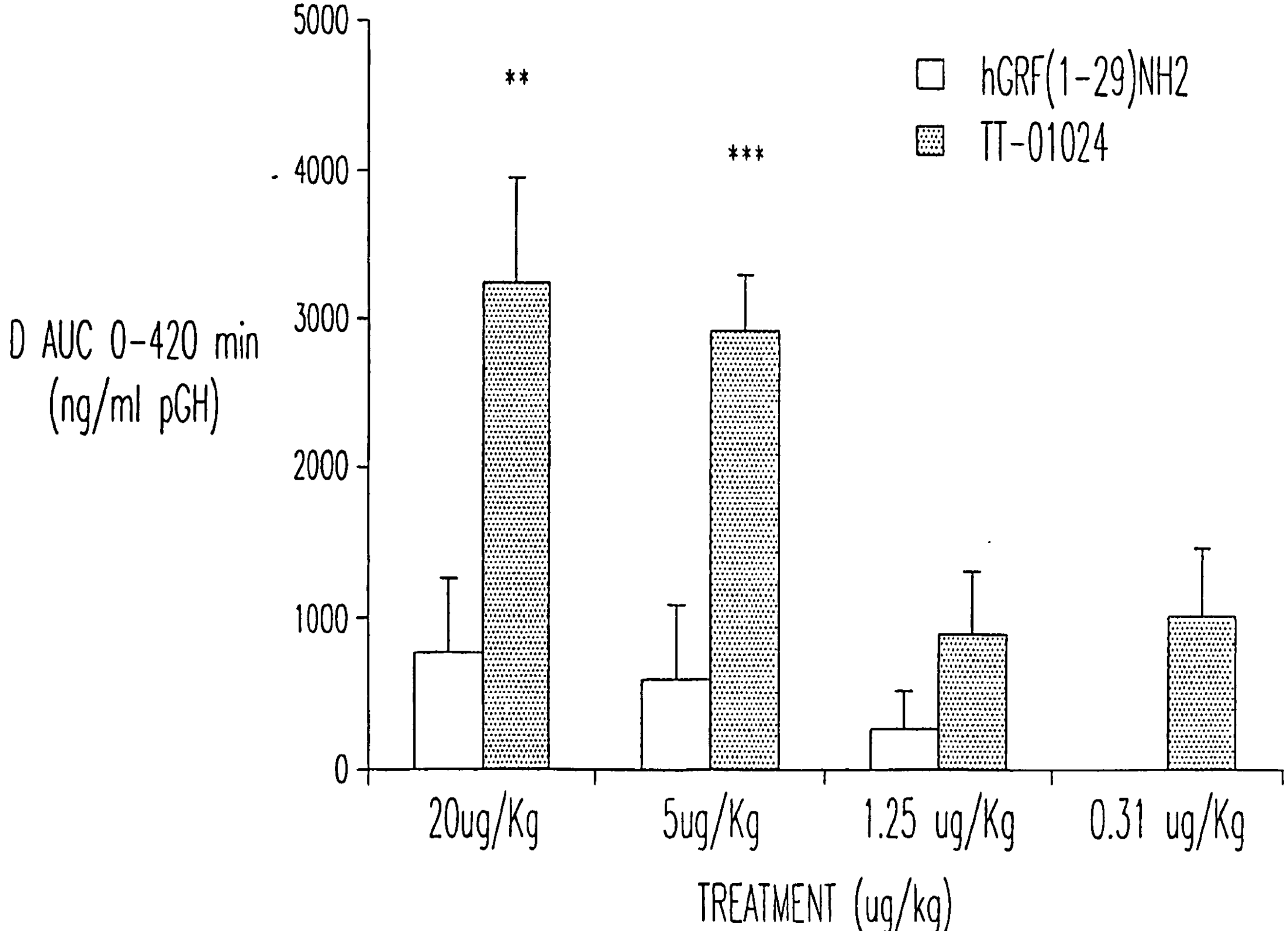

hGRF(1-29)NH2
TT-01024
D AUC 0-300min
(ng/ml pGH)
5000
4000
3000
2000
1000
0
***
***
***
**
4ug/Kg
1ug/Kg
0.25 ug/Kg
TREATMENT

FIG. 3



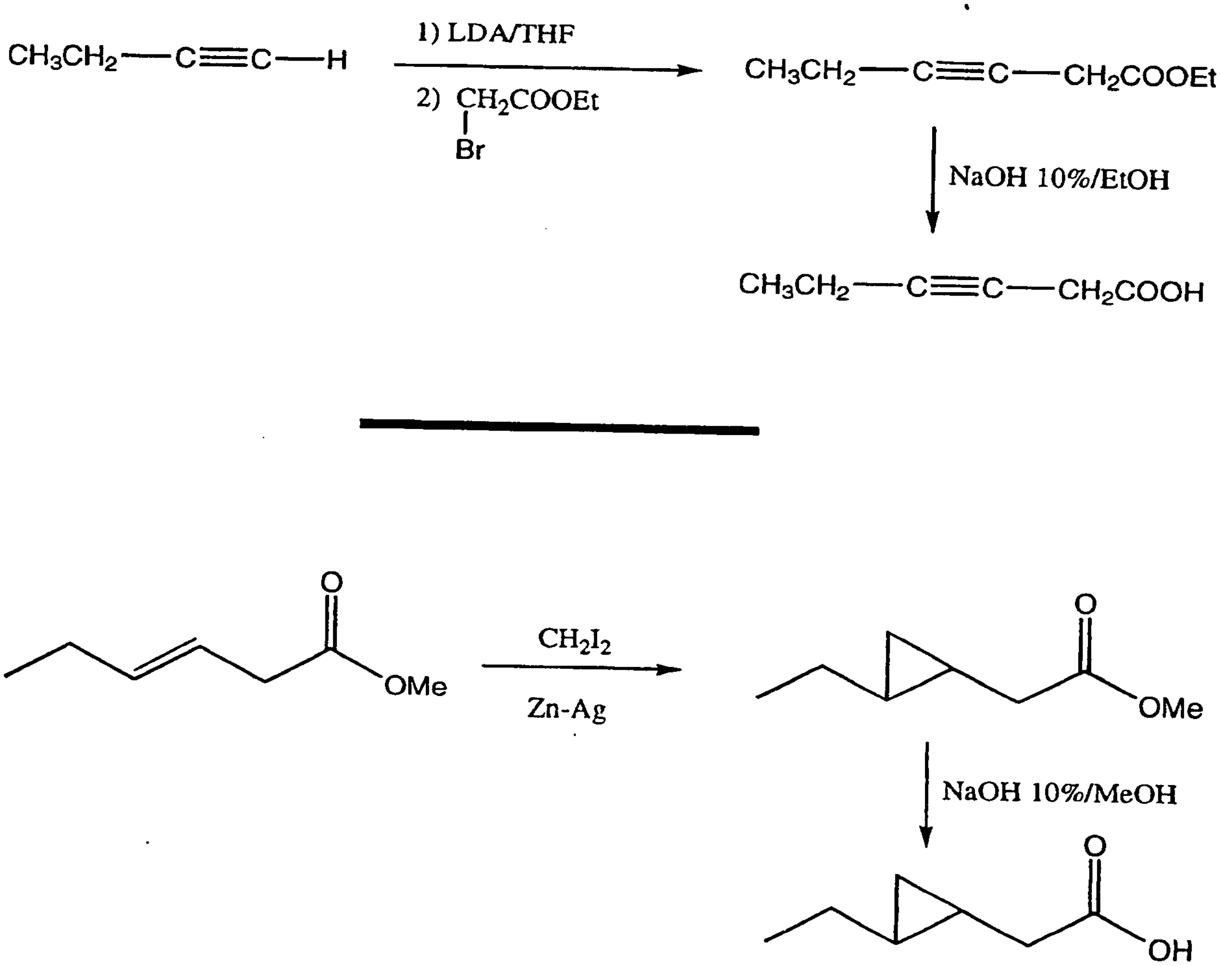
GH(ng/ml)
30
25
20
15
10
5
0
-60 -40 -20 0 20 40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360 380 400 420
Minutes
Saline
hGRF(1-29)NH2 5ug/Kg
TT-01024 5 ug/kg

FIG. 4



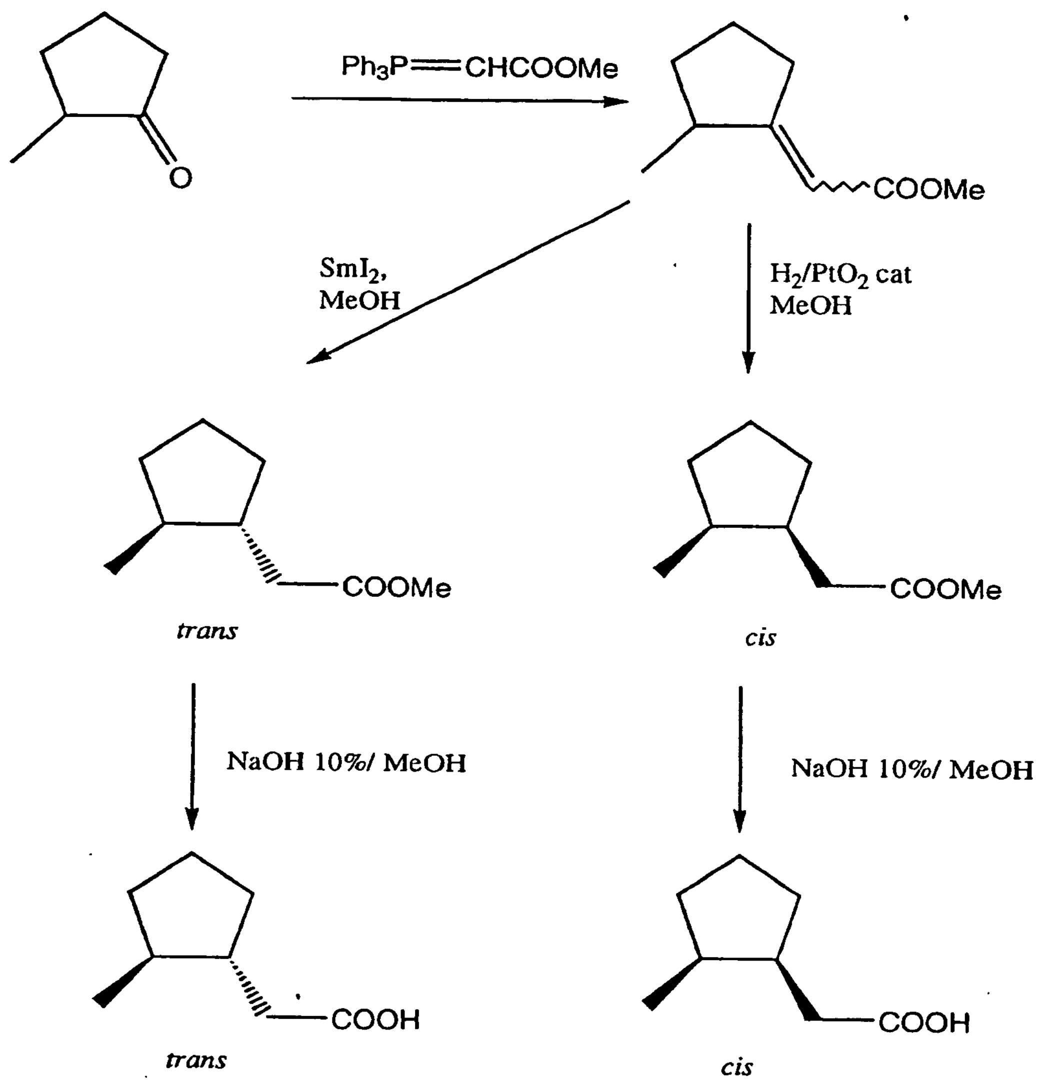
D AUC 0-420 min
(ng/ml pGH)
5000
4000
3000
2000
1000
0
**
***
hGRF(1-29)NH2
TT-01024
20ug/Kg
5ug/Kg
1.25 ug/Kg
0.31 ug/Kg
TREATMENT (ug/kg)

FIG. 5



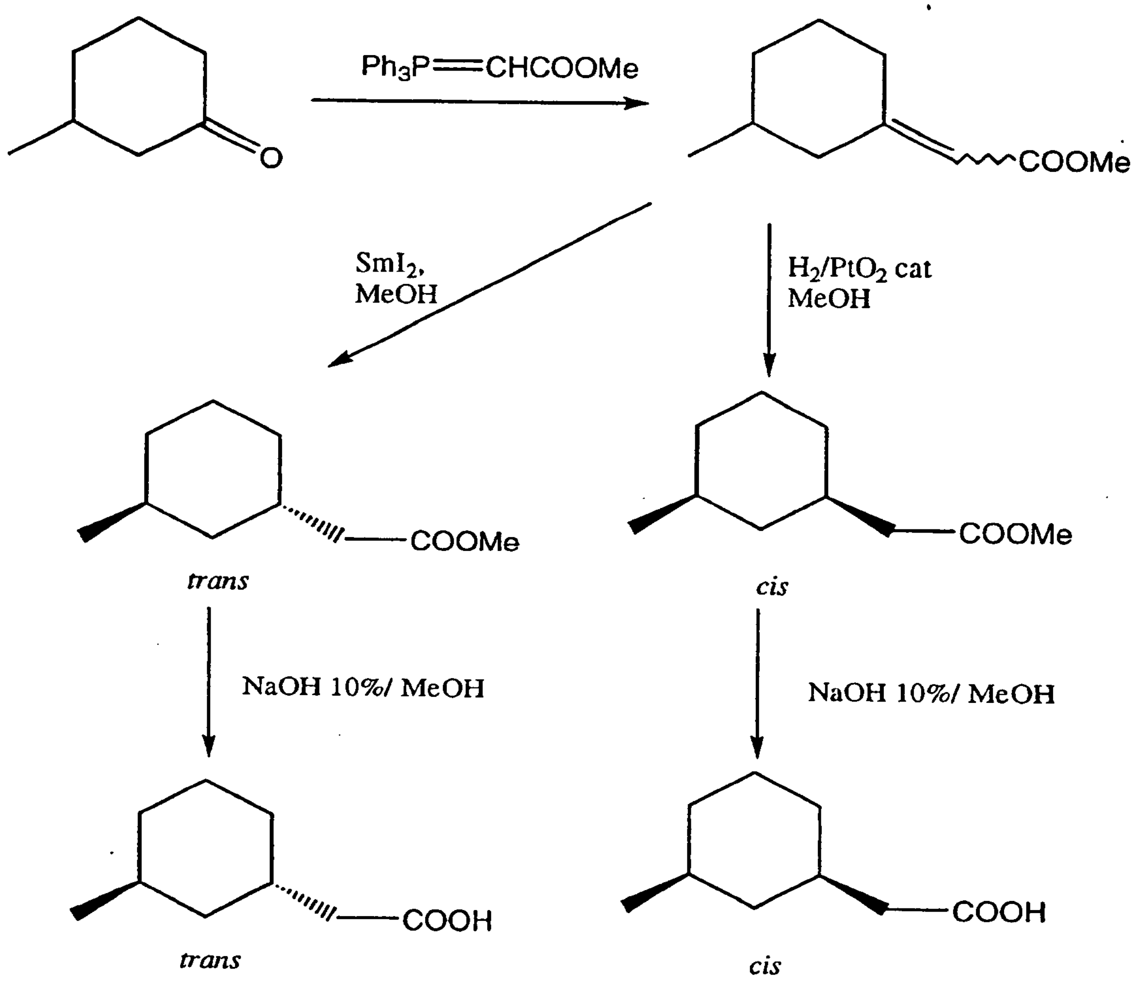
$CH_3CH_2—C≡C—H$
1) LDA/THF
2) CH_2COOEt | Br
$CH_3CH_2—C≡C—CH_2COOEt$
NaOH 10%/EtOH
$CH_3CH_2—C≡C—CH_2COOH$
O
OMe
CH_2I_2
Zn-Ag
O
OMe
NaOH 10%/MeOH
O
OH

Fig. 6A

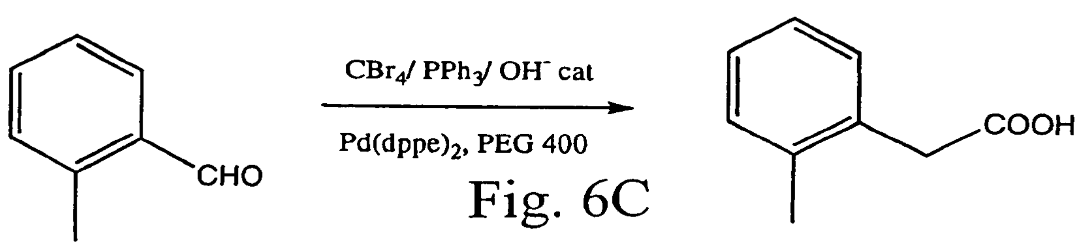


Ph3P═CHCOOMe
O
COOMe
SmI2,
MeOH
H2/PtO2 cat
MeOH
COOMe
trans
COOMe
cis
NaOH 10%/ MeOH
NaOH 10%/ MeOH
COOH
trans
COOH
cis

Fig. 6B



Ph3P=CHCOOMe
O
COOMe
SmI2, MeOH
H2/PtO2 cat MeOH
COOMe
trans
COOMe
cis
NaOH 10%/ MeOH
NaOH 10%/ MeOH
COOH
trans
COOH
cis



CHO
CBr4/ PPh3/ OH- cat
Pd(dppe)2, PEG 400
COOH

Fig. 6C